Nuclear Magnetic Resonance Studies Of Free Fatty Acid Induced Insulin Resistance In Vivo

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Abstract

NUCLEAR MAGNETIC RESONANCE STUDIES OF FREE FATTY ACID INDUCED INSULIN RESISTANCE IN VIVO.

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Elevated levels of plasma free fatty acids (FFA) have been shown to cause a decrease in the rate of insulin-stimulated glucose uptake in muscle. However, the mechanism by which this occurs is unclear. In order to clarify the effects of acute increases of FFA on glucose disposal *in vivo*, we used ^{13}C NMR and the euglycemic (5mM) /hyperinsulinemic (10mU/kg/min) clamp in awake rats with or without a five hour preinfusion of lipid/heparin. Elevated plasma FFA caused a significant decrease in the rate of whole body glucose disposal, as reflected by the ~35% drop in glucose infusion rate ($p< 0.005$). This decrease was associated with a 40-50% reduction in the rate of muscle glycogen synthesis ($p < 0.01$) and muscle glucose oxidation ($p < 0.005$). In a separate experiment, the five hour pre-infusion of lipid was followed by a euglycemic/ hyperinsulinemic clamp using 2-[1,2-^{3}H] deoxyglucose to assess rates of glucose transport activity in vivo. In the group with high FFA, glucose transport activity was decreased ~25% compared to controls ($p < 0.05$). We conclude that acute elevations of FFA for five hours cause insulin resistance in the skeletal muscle of rats in vivo through a reduction in insulin-stimulated muscle glycogen synthesis and glucose oxidation. This reduction is associated with a decrease in glucose transport activity.

Acknowledgments

This work is dedicated to the 16 million Americans who have diabetes. It is my hope that this project will contribute to the understanding of the pathogenesis of this potentially devastating disease, offer insight into treatment options, and ultimately better the lives of our patients who suffer from this illness and its complications.

My deepest gratitude goes to

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and God, who has guided me throughout my life and in whom I have eternal hope.

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Introduction

Non-insulin dependent diabetes mellitus is often associated with obesity and/or elevated levels of plasma free fatty acids (FFA).[1,2] In 1963, Randle et al demonstrated that free fatty acids compete effectively with glucose for substrate oxidation at the level of isolated rat heart and diaphragmatic muscle.[3] As Figure 1 illustrates, Randle proposed that FFA undergo B-oxidation which increases the intra-mitochondrial acetyl-CoA/CoA and NADH/NAD+ ratios. These increased ratios lead to an inhibition of pyruvate dehydrogenase activity which elevates levels of citrate. Since citrate is an important allosteric inhibitor of phosphofructokinase, glucose-6-phosphate builds up which in turn inhibits hexokinase II. The subsequent rise in concentration of intracellular glucose diminishes the glucose gradient, impairs glucose transport, and results in hyperglycemia.

Whether the Randle cycle, or glucose-free fatty acid cycle, operates in skeletal muscle has been the subject of intense investigation with mixed findings. Studies in healthy humans and rats have revealed that lipid/heparin infusions, which raise plasma FFA levels, do in fact induce insulin resistance under conditions of hyper- and euglycemic hyperinsulinemia[4,5,6] and impair insulin-dependent glucose uptake by human forearm tissues.[7] The data from these studies revealed a FFA-induced defect in glucose oxidation, a finding consistent with Randle's hypothesis. However, subsequent studies challenged this idea and found no inhibitory action of FFA on nonoxidative glucose metabolism.[8,9]

Recent work by Boden et. al.[10,11] demonstrated that a reduction in carbohydrate oxidation could only account for one-third of the fatty acid induced drop in glucose uptake. Rather, a decrease in glycogen synthesis accounted for the majority of the drop in glucose uptake in skeletal muscle. At plasma FFA concentrations $\geq$ 0.75mM in humans, Boden et. al. found increased levels of muscle glucose-6-phosphate (through muscle biopsies) which they interpreted as FFA induced defect in glycogen synthase activity.[10] This interpretation

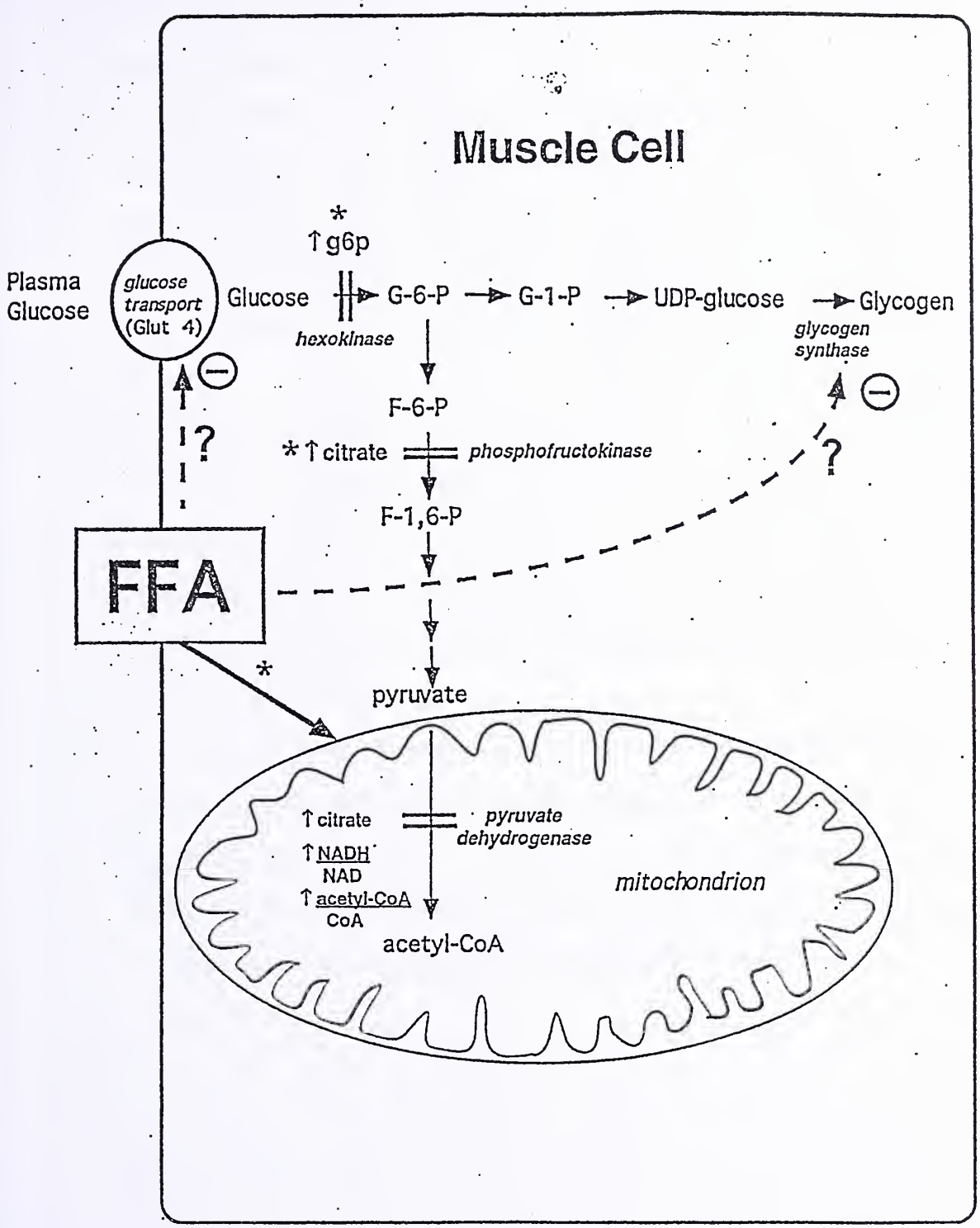


Figure 1: Schema of potential sites of free fatty acid action on insulin mediated glucose metabolism in skeletal muscle and those sites (*) hypothesized to be affected by Randle et al.[3] g6p, glucose-6-phosphate; G-1-P, glucose-1-phosphate; F-6-P, fructose-6-phosphate; F-1,6-P, fructose-1,6 biphosphate. Figure taken from paper by Boden et al.[4]

has not been supported by other studies which found no impact of fat on glycogen synthase activity.[9,12] However, as pointed out by Roden et al[13], the interpretation of results based on enzyme activities measured in vitro has several limitations a) muscle biopsies offer only several time points in the experiment b) enzyme activities may not necessarily reflect in vivo substrate flux and c) measurements of glucose-6-phosphate in muscle biopsies can be falsely elevated due to glycogen breakdown between sample excision and freezing.[13]

With the advent of NMR (nuclear magnetic resonance) spectroscopy, one can noninvasively and continuously measure key metabolites in glucose metabolism *in vivo* and overcome these limitations. Our group has recently found through ^{31}P NMR spectroscopy, that increased plasma FFA concentrations of ~1.8mM result in a decrease, not an increase as Boden et. al. found, in glucose-6-phosphate (G-6-P) concentrations in the skeletal muscle of humans.[14] The decrease in [G-6-P] is more consistent with a defect in transport/phosphorylation activity than with a defect in glucose oxidation secondary to increased FFA B-oxidation.

We set off to develop an animal model to better appreciate the mechanism by which FFA impair insulin-mediated glucose uptake in skeletal muscle *in vivo*. Using ^{13}C NMR spectroscopy and the euglycemic/hyperinsulinemic clamp, we studied the effects of FFA on muscle glycogen synthesis, glucose oxidation, and glucose transport activity with or without a preinfusion of lipid/heparin in awake Sprague-Dawley rats.

Statement of Purpose

We set off to develop an animal model to better appreciate the mechanism by which FFA impair insulin-mediated glucose uptake in skeletal muscle *in vivo*. Using ^{13}C NMR spectroscopy and the euglycemic/hyperinsulinemic clamp, we studied the effects of FFA on muscle glycogen synthesis, glucose oxidation, and glucose transport activity with or without a preinfusion of lipid/heparin in awake Sprague-Dawley rats.

Methods

Male Sprague-Dawley rats (Charles River, Raleigh, NC) weighing between 250 and 350g were maintained on standard rat chow (Ralston Purina, St. Louis, MO) and housed in an environmentally controlled room with a 12:12-h light-dark cycle. Rats were chronically catheterized in the right jugular vein and carotid artery and the catheters were externalized through a skin incision at the back of the head.[15] The rats were allowed to recuperate after surgery until they were at least of preoperative weight (5-7 days). All rats were fasted 15-18 h before each infusion study. The rats were transiently anesthetized (<30s) with a low dose (2.5 mg) of thiopental (Sigma Chemical, St. Louis, MO) and placed in a restraining tube modified to allow one hindlimb to be secured to the outside of the tube. The restraining tube was then mounted to the radiofrequency (RF) coil assembly such that the rat hindlimb was centered over the surface coils. The rat and RF coil assembly were then placed in the bore of the magnet, with the hindlimb positioned at the magnet isocenter. Infusion lines were extended to syringes and pumps outside the RF enclosure of the magnet. After collection of a 15 minute baseline spectrum, acquisition of spectra were made continuously according to one of two protocols described below.

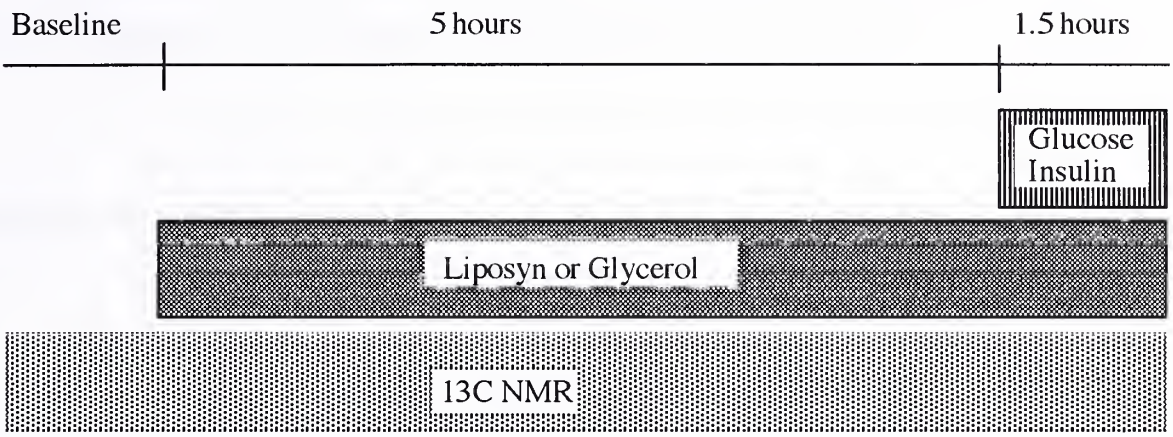
Protocol 1

Two groups of Sprague-Dawley rats prepared as previously described were studied using a 5 hour pre-infusion protocol of lipid/heparin (n=6) or glycerol (n=4) as shown in Figure 2. Liposyn II (Abbott Laboratories), a 20% triglyceride emulsion (10ml/kg/hr) combined with heparin (0.0975 IU/min) or glycerol (1:3 v/v, 0.425 mg/min) was continuously infused. Heparin was used to activate lipoprotein lipase which served to breakdown the triglycerides in the Liposyn infusion to FFA. A 90 minute hyperinsulinemic-euglycemic clamp was begun utilizing a 1-^{13}C enriched variable 20%

dextrose infusion (99% enriched, 20% w/v, Cambridge Isotope Laboratories, Cambridge, MA) during which the lipid or glycerol infusion continued. By drawing blood samples every 5 minutes and determining the plasma glucose concentration (Glucose Analyzer II; Beckman Instruments, Fullerton, CA), we could vary the glucose infusion rate and maintain a glucose concentration of approximately 5.5 mM. During the clamp glycogen synthesis rates were measured using ^{13}C NMR with a 7 Tesla magnet in the awake rats according to established methods.[16] Following the infusion the superficial skin over the left hindquarter was removed and mixed gastrocnemius muscle was frozen in *situ* .

Using the technique developed by Bloch et. al.[17], glycogen synthetic rates were obtained. Briefly, the incremental change in C-1 glycogen peak intensity from [1-^{13}C] glucose incorporation was measured at 100.5 ppm. Incremental plasma glucose ^{13}C fractional enrichment as well as final glycogen ^{13}C enrichment and concentrations were used to back extrapolate the glycogen concentration at each measured time point to baseline. Applying a linear regression analysis over these timepoints yielded the glycogen synthetic rate. Steady state pyruvate dehydrogenase (PDH) flux to tricarboxylic acid (TCA) flux was determined from the ratio of ^{13}C enrichment of C3 alanine to C4 glutamate and the relative amount of intracellular pyruvate derived from plasma glucose was estimated by the ^{13}C enrichment in C3 alanine (muscle) to C1 plasma glucose x 0.5 as previously described.[7]

Figure 2: Experimental Design



Protocol 2

Two groups of Sprague-Dawley rats were infused with either glycerol (n=7) or lipid/heparin (n=5) for five hours. After preinfusion, all rats underwent a hyperinsulinemic-euglycemic clamp. Skeletal muscle glucose uptake was measured according to methods described by Kraegen et al.[18] Briefly, thirty minutes after the insulin/glucose infusion was begun, 40mCi of 2-[1,2-^{3}H] deoxy-D-glucose ([^{3}H]-2DG) was injected as a bolus. Plasma samples were obtained at frequent intervals up to 45 minutes following the bolus infusion to estimate plasma tracer activity. At 45 minutes, the animals were anesthetized and mixed gastrocnemius muscle was freeze-clamped and excised. Glucose uptake rate calculations were based upon mean plasma glucose and tissue [^{3}H]-2DG and the area under the plasma [^{3}H]-2DG curve as described by Kraegen et al.[6] Muscle [^{3}H]-2DG concentrations were measured according to Nguyen et al.[19]

Analytical Procedures

Measurements of free insulin, plasma FFA, C13 enriched glucose, alanine, and glycogen were done by our laboratory technicians as previously described.[16]

Statistical Analysis

All data are reported as the mean ± SE. Student's two tailed t-test was performed on data to determine significance at a minimum of $p \leq 0.05$ threshold.

Results

In protocol 1, the plasma glucose concentrations were clamped at approximately 5.5 mM in both the control and high plasma FFA groups, with no significant difference between the two groups. Glucose infusion rates were reduced by 34% when plasma FFA were increased by the lipid infusion (Table I). NMR studies confirmed insulin resistance at the level of skeletal muscle as reflected by ~40% reduction in muscle glycogen synthetic rates during the hyperinsulinemic-euglycemic clamp following lipid infusion compared to the control glycerol infusion. The ratio of PDH to TCA cycle flux, reflecting entry of pyruvate relative to other substrates (mainly FFA) into the TCA cycle fell from 0.49±0.01 in the control group to 0.23±0.01 in the lipid-infused rats. This decreased PDH/TCA ratio reflects relative decreased muscle glucose oxidation since most (~70%) of the intracellular pyruvate was derived from plasma glucose in both groups as reflected by the $^{13}C3$ alanine (tissue)/ $^{13}C1$ glucose (plasma) ratio x 0.5 (controls 0.74 ± 0.01, lipid 0.70 ± 0.01).

The rats studied during the [^{3}H]-2DG clamps were matched with regard to basal levels of FFA and basal insulin concentrations similar to the NMR experiment. Rats which were preinfused with lipid had significantly higher levels of FFA during the clamp than the glycerol infused rats (3.3±0.8 mmol/l vs 0.6±0.2 mmol/l). Again this elevation in plasma FFA concentration was associated with insulin resistance as demonstrated by a 40% reduction in glucose infusion rate (from 237±18 umol/kg/min in the glycerol infused rats to 141± 22 umol/kg/min in the lipid infused animals). [^{3}H]-2DG uptake in skeletal muscle of lipid infused rats was 8.8±0.4 umol/100g muscle/min compared to glycerol infused rats uptake of 11.4±1.0 umol/100g muscle/min ($p<0.05$) despite comparable levels of hyperinsulinemia during the clamp (3324±1428 pmol/l vs 2814±618 pmol/l).

Table 1: Metabolic Data from Hyperinsulinemic-Euglycemic Clamp Studies

	Glycerol	Lipid	
FFA during clamp: protocol 1 (mmol/L)	0.4±0.1	2.8±0.7	$p < 0.05$
FFA during clamp: protocol 2 (mmol/L)	0.6±0.2	3.3±0.8	$p < 0.05$
Glucose Infusion Rate (umol/kg/min)	244±22	161±10	$p < 0.005$
Glycogen Synthesis Rate (umol/kg/min)	147±14	80±11	$p< 0.01$
PDH flux/ TCA flux	0.49±0.01	0.23±0.01	$p< 0.005$
2-[1,2-^{3}H] deoxyglucose (uM/100g muscle/min)	11.4±1.0	8.8±0.3	$p<0.05$

Discussion

Acute elevations in plasma FFA concentrations for five hours induced insulin resistance in this awake rat model as reflected by ~35% reduction in the glucose infusion rate during the hyperinsulinemic-euglycemic clamp. The drop in insulin-mediated glucose uptake could be largely attributed to the ~45% reduction in the rate of muscle glycogen synthesis. These findings agree with a recent study by Chalkley et al.[20] We also discovered a ~50% reduction in the relative rate of muscle glucose oxidation as shown by the decrease in the relative ^{13}C enrichment in the C4 position of muscle glutamate versus the C3 position of intramuscular alanine. The combined decrease in both muscle glycogen synthesis and glucose oxidation suggest that FFA have a more global effect on metabolism than Randle's model would have predicted. In fact, the decrease in both oxidative and nonoxidative glucose metabolism is consistent with a FFA induced defect in glucose/transport phosphorylation activity. Support of this view is provided by results from protocol 2 which revealed lower rates of [^{3}H]-2 deoxyglucose uptake in the lipid infused animals than in the glycerol controls.

Still, even if one were to reject Randle's hypothesis and accept the premise that FFA induce insulin resistance by impairing glucose transport/phosphorylation, the question still remains precisely how FFA are exerting their effects. Some investigators have turned to the insulin signaling cascade for answers. PI3 kinase is a key regulator of Glut4 translocation in muscle leading to increased glucose transport and its activity is reduced in muscle strips taken from subjects with type 2 diabetes[21] and obesity[22]- diseases which are associated with chronically elevated levels of FFA. In fact, studies from our lab using the *same* protocols described previously have shown that acutely elevated plasma FFA reduce PI3 kinase activity by over 50%[23].

In conclusion, contrary to the glucose-free fatty acid cycle proposed by Randle et. al.[3], elevated plasma free fatty acids cause a decrease in insulin stimulated glucose uptake

by inhibiting both muscle glycogen synthesis and glucose oxidation which would be more consistent with a defect in glucose transport/phosphorylation than with a initial FFA inhibition of pyruvate dehydrogenase. Glucose transport activity was in fact decreased in the lipid infused rats. Further studies will be needed to elucidate the precise signaling defects associated with/ caused by elevated FFA.

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